

Combined inhibition of complement and CD14 in human experimentally induced bacterial inflammation and in porcine sepsis

PhD Thesis

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LIST OF PAPERS

Paper I

Egge KH, Barratt-Due A, Nymo S, Lindstad JK, Pharo A, Lau C, Espevik T, Thorgersen EB, Mollnes TE. The anti-inflammatory effect of combined complement and CD14 inhibition is preserved during escalating bacterial load. Clin Exp Immunol. 2015 Apr. *In press*

Paper II

Egge KH, Thorgersen EB, Lindstad JK, Pharo A, Lambris JD, Barratt-Due A, Mollnes TE. Post-challenge inhibition of C3 and CD14 attenuates *Escherichia coli*-induced inflammation in human whole blood. Innate Immun. 2014 Jan; 20(1):68-77.

Paper III

Barratt-Due A, Thorgersen EB, **Egge K**, Pischke S, Sokolov A, Hellerud BC, Lindstad JK, Pharo A, Bongoni AK, Rieben R, Nunn M, Scott H, Mollnes TE. Combined inhibition of Complement (C5) and CD14 markedly attenuates inflammation, thrombogenicity and hemodynamic changes in porcine sepsis. J Immunol. 2013 Jul; 191(2):819-27.

Paper IV

Egge KH, Thorgersen EB, Pischke SE, Lindstad JK, Pharo A, Bongoni AK, Rieben R, Nunn MA, Barratt-Due A, Mollnes TE. Organ inflammation in porcine *Escherichia coli* sepsis is markedly attenuated by combined inhibition of C5 and CD14. Immunobiology. 2015 May. *In press*.

ABBREVIATIONS

Ab	Antibody
ANOVA	Analysis of Variance
AU	Arbitrary Units
AP	Alternative Pathway
BK	Bradykinin
C	Complement factor
CD	Cluster of Differentiation
CLP	Cecal Ligation and Puncture
CP	Classical Pathway
CR	Complement Receptor
CRP	C-Reactive Protein
CVP	Central venous pressure
C5aR	C5a Receptor
DAMP	Damage-Associated Molecular Pattern
DIC	Disseminated Intravascular Coagulation
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
FITC	Fluorescein Isothiocyanate
ICU	Intensive Care Unit
Ig	Immunoglobulin
IL	Interleukin
kPa	KiloPascal
LP	Lectin Pathway
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MAP	Mean Arterial Pressure
MBL	Mannose Binding Lectin
MD-2	Myeloid Differentiation factor 2
MFI	Median Fluorescence Intensity
mg	Milligram
mL	Milliliter

mM	Millimolar
mmol	Millimol
MPAP	Mean Pulmonary Arterial Pressure
MyD88	Myeloid Differentiation Primary Response Gene 88
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
OmCI	<i>Ornithodoros moubata</i> Complement Inhibitor
PAI-1	Plasminogen Activator Inhibitor 1
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
SIRS	Systemic Inflammatory Response Syndrom
SVRI	Systemic Vascular Resistance Index
TAT	Thrombin-Antithrombin Complex
TCC	Terminal Complement Complex
TF	Tissue Factor
TNF	Tumor Necrosis Factor
TLR	Toll-Like Receptor
TRIF	TIR-domain-containing Adapter-inducing Interferon-β
WBC	White Blood Cell Count

1. INTRODUCTION

1.1 Sepsis

Sepsis is Greek for putrefaction or decay. In clinical medicine it is a term used to describe the systemic response to an infection, defined as “a systemic inflammatory response syndrome (SIRS) caused by infection” (1). By the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference in August 1991, SIRS was originally defined by the presence of more than one of the following criteria: (I) a body temperature greater than 38°C or less than 36°C; (II) a heart rate above 90 beats/min; (III) a respiratory rate greater than 20 breaths/min or hyperventilation defined by PaCO₂ less than 4.3 kPa; and (IV) an alteration in the white blood cell count (WBC) defined by WBC greater than 12 x 10⁹ /L or WBC less than 4 x 10⁹ /L or more than 10% immature neutrophils in the WBC (1). Later reviews of the SIRS definition have made slight alterations to the original criteria and taken in several more criteria including inflammatory, haemodynamic, organ dysfunction and tissue perfusion variables (2,3). As a number of non-infectious diseases, such as trauma, burns and pancreatitis may also cause SIRS, a documented or suspected infection is a diagnostic requirement for sepsis (3).

Based on severity, sepsis is stratified into four subgroups: (I) sepsis; (II) severe sepsis; (III) septic shock; and (IV) sepsis with multiple organ dysfunction syndrome (MODS) (1). In the US sepsis and severe sepsis are the most common causes of death among critically ill patients in non-coronary intensive care units (ICU), and overall one of the leading causes of death (4). These conditions are public health problems of great cost (5). As growing patient groups such as elderly patients and patients with pre-existing chronic diseases are at increased risk, the public health care issues and death tolls may most possibly increase in the future (4). It would therefore be of great significance to find more effective means to combat the disease.

The leading causes of sepsis in the US and Europe are almost equally divided between Gram-positive and Gram-negative infections, although with an increase in Gram-positive infections over the last few decades (6-8). The vast majority of the Gram-positive infections are caused

by a variety of *Staphylococci* species, and in a large European multicentre ICU study from 2006 (the SOAP study), species of *Staphylococci* and *Streptococci* accounted for more than 90 % of all Gram-positive infections in septic ICU patients (6). With regards to the Gram-negative infections in the same study, *Escherichia coli* and *Pseudomonas* species were found to be causative in over 50 % of cases (6). Just as interesting, the same study reports that as much as 40 % of all septic ICU patients only had clinical signs of infection, and no causative microbe was found. Furthermore, as much as 18 % of the septic patients had polymicrobial infections (6). The findings of the SOAP study are analogous to previous and later studies (7,8).

1.2 Innate immunity and inflammation

The body is under constant threat from microbes in its natural environment. Under physiological circumstances, the microbes are held outside the body by the skin and mucosal surfaces as a first line barrier against infections (9). As soon as the microbes trespass the barrier, the immune system is set in action (9). In the course of evolution, the immune system has developed several mechanisms in order to cope with invading microbes. These mechanisms are commonly divided into two main branches: innate and adaptive immunity. Adaptive immunity serves as the “long term” memory of the immune system. Its main function is to remember specific microbes with strong affinity in order to detect and destroy them whenever encountered (9).

Innate immunity on the other hand, is functional even without previous sensitization and depends on germ-line encoded receptors, referred to as pattern recognition receptors (PRRs) (10). The cardinal trigger of the immune response is the recognition of conserved molecular patterns of exogenous as well as endogenous molecules by these PRRs (11,12). The exogenous conserved patterns from different microbes are referred to as pathogen-associated molecular patterns (PAMPs), whereas the endogenous conserved patterns, which do not originate from microbes but rather from damaged host cells, are referred to as damage-associated molecular patterns (DAMPs) (13-15). Thus, innate immunity is not only structured to combat invading pathogens, it is also designed to detect, repair or clear damaged cells,

representing danger from within the host itself. The main goal of innate immunity, whether it detects microbes or damaged self, is to re-establish homeostasis (10).

Confusingly, in the "danger model" proposed by Polly Matzinger, the abbreviation DAMPs is used as short for danger-associated molecular patterns, a common denomination for PAMPs and alarmins (15). This model reflects the view that innate immunity is designed to detect danger, exogenous (PAMPs) and endogenous (alarmins), rather than discriminate self from non-self, which in the 1990ies was the competing view on innate immunity as stated by Charles Janeway in his "self-non-self" model (16). In this thesis DAMPs will be used as short for damage-associated molecular patterns and not as a common denomination for PAMPs and alarmins.

Upon recognition of PAMPs and DAMPs via PRRs, innate immunity responds by inducing inflammation (10). The inflammatory mediators are secreted by a number of cells, including macrophages, monocytes, granulocytes and dendritic cells of the immune system (12,17). These mediators are commonly denominated cytokines and include a broad variety of locally and systemically active molecules, which induce chemotaxis, regulate expression of cell surface receptors and adhesion molecules, increase capillary leakage, trigger coagulation and fibrinolysis, and activate macrophages and fibroblasts, to mention some of its multifaceted activities (18). In addition a wide variety of other mediators such as prostaglandins and leukotrienes directly or indirectly induce a diverse inflammatory response (19).

This thesis is mainly focused on PAMPs. Importantly however, DAMPs and PAMPs appear largely to depend on the same PRRs to evoke inflammatory responses (9). Therefore, the translational value of research on pathogen-induced inflammation to sterile-induced inflammation may prove to be of great significance.

PRRs of innate immunity involved in detection of PAMPs include several groups of receptors: Intracellular receptors, cell surface bound receptors and soluble receptors (17). Examples of intracellular PRRs are Nucleotide Oligomerization Domain-like (NOD-like) receptors and

Retinoic acid Inducible Gene-like (RIG-like) receptors which recognize intracellular microbial PAMPs and intracellular viral RNA respectively (20-23). To the family of exclusively intracellular PRRs belong also the relatively newly discovered AIM-like (Absent In Melanoma-like) receptors (23). These intracellular PRRs and their effector functions will not be discussed in this thesis.

Toll-like receptors (TLRs) including its soluble co-receptors and the complement system are important examples of membrane bound and soluble PRRs (17). They play key roles in the network of innate immunity functioning as central upstream sensor- and effector-systems, thus to a large part being responsible for the complex and widespread inflammatory response (15,24). These systems, their role and the interplay between them in Gram-negative and Gram-positive induced inflammation, and partly also in sterile induced inflammation will be described in the following.

1.3 The complement system

The complement system is an important part of innate immunity, where it plays a vital role defending the host against pathogens and maintaining homeostasis (25). Over the last decades it has become clear that it serves as a bridge to adaptive immunity involving T and B cells (26), and also is involved in the pathogenesis of an increasing number of conditions where microbes are not involved (27). It is constituted by over 30 different plasma and cell surface proteins forming a cascade system with a plethora of physiological effects (28,29). Activation of the cascade is triggered via one of three possible pathways; the classical, the lectin and the alternative pathways. All pathways converge on the central complement factor C3 which is cleaved to C3a and C3b, thereby launching the terminal pathway as C5 is cleaved to C5a and C5b and the assembly to form the terminal complement complex is initiated (TCC) (25,30).

The classical pathway (CP) is initiated when immune complexes are formed from C1q binding to antibodies in complex with pathogenic surface antigens or other non-self antigens (30). In addition, pentraxins, such as the acute phase protein CRP, may clear pathogens by directly binding to C1q (31). The complex recruits C1r and C1s, the latter cleaving C4 and C2 to form C4bC2b – the C3 convertase of the CP.

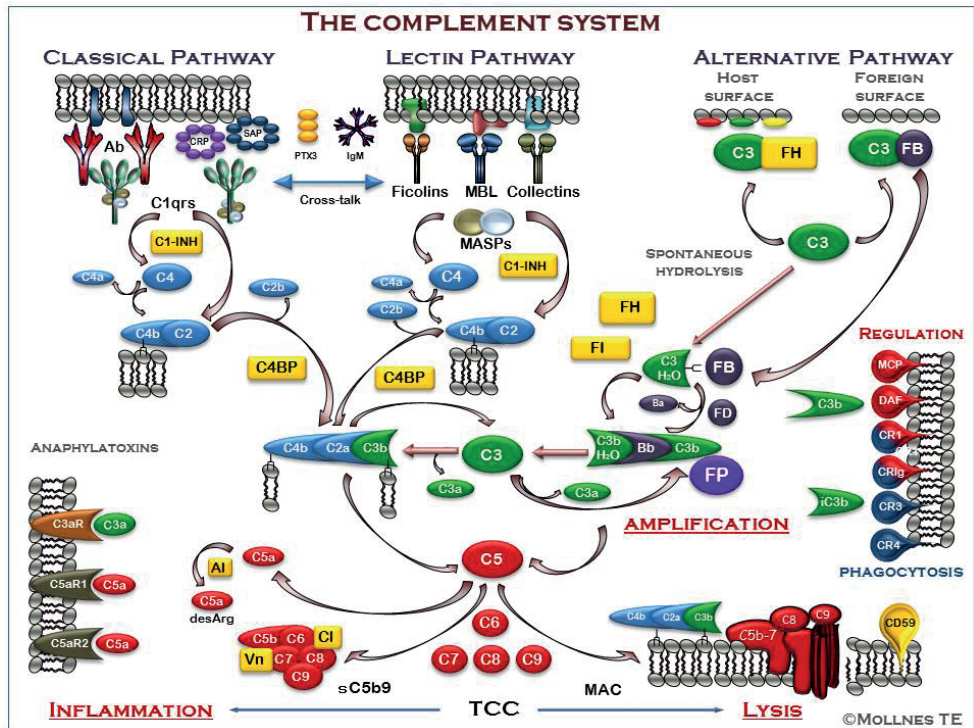


Figure 1. The complement system (with permission from the copyright holder T. E. Mollnes).

The lectin pathway (LP) is activated when mannose binding lectin (MBL), ficolins or collectins are bound to carbohydrate structures on the surface of pathogens (25). In the circulation, MBL, ficolins and collectins are bound to MBL-associated proteins (MASPs) (32), which undergo conformational changes upon binding to pathogens, thereby inducing cleavage of C4 and subsequent recruitment and cleavage of C2 to ultimately form C4bC2b – the C3 convertase of the LP.

The alternative pathway (AP) has a “spontaneous tick over”, as hydrolysis of C3 to form C3(H₂O) goes on continuously (33). In addition, the pathway may be triggered by several molecular structures, including carbohydrates, proteins and lipids on pathogens and other non-self surfaces which split C3 into C3b and the anaphylatoxin C3a. Further activation takes place as C3(H₂O) or C3b are bound to factor B, which is further cleaved by factor D to form

C3(H₂O)Bb or C3bBb respectively – both C3 convertases of the AP. These convertases are further stabilized when they form complex with Properdin (or factor P) (33). Properdin has previously also been regarded as a PRR of the AP by recruitment of C3b to induce assembly of the AP C3 convertase (25,33), but results from later studies seriously question the role of Properdin as a PRR (34). In addition to being an activation pathway, the AP also functions as an essential amplification loop for the CP and the LP, maybe responsible for as much as 80% of the final formation of C5a and TCC (34,35).

Following the cleavage by the C3 convertases, C3b forms complexes with the same C3 convertases C4bC2b and C3bBbP, producing the C5 convertases C4bC2bC3b and C3bBbPC3b. These convertases cleave C5 to C5a and C5b, initiating the mentioned assembly of the TCC as C6 and C7 bind to C5b followed by C8 and lastly multiple C9 molecules (25).

When multiple TCCs are inserted into the membrane of certain microbes and cells, each of the TCCs form a pore, and ultimately lysis may occur. In this setting TCC is often referred to as the membrane attack complex (MAC). This mechanism is especially important in the defence against infections with *Neisseria (N.) meningitides*, one of the microbes responsible for causing meningitis (36). As importantly, when TCCs are formed in sublytic concentrations on membranes, they may induce proinflammatory responses, for instance on endothelial cells and other nuclear cells (37).

Another and important way the complement system defends the host from invading pathogens is by opsonisation of the microbes by deposition of C3b and C4b fragments to induce phagocytosis (38). In addition to bactericidal activity, it is also responsible for tissue regeneration and clearance of apoptotic cells and debris (39,40), but more importantly, especially in the setting of sepsis, it is one of the main inflammatory triggers.

The small split fragments of the complement cascade C3a and C5a are highly biologically active anaphylatoxins and the main inflammation inducing complement products (41-43). C3a and C5a have several biological functions in common such as promotion of vasodilation and

increasing capillary leakage (44), and both are associated with pathogenesis in asthma and acute respiratory distress syndrome (ARDS) (45). C3a also induces mast cell degranulation and transcription factor activation thereby increasing inflammation (46). Of the two, C5a is a particularly potent inflammation inducer and a plethora of effects includes upregulation of adhesion molecules, stimulation of phagocytosis and oxidative burst in neutrophils and monocytes, induction of granular enzyme release and attraction of neutrophils to the site of danger (42,44,47). On the other hand, recent studies show that C3a also has several anti-inflammatory effects partly opposing the proinflammatory effects of C5a, a noteworthy point in the debate about where in the complement cascade to intervene therapeutically (48).

All of the above mentioned effects are favourable under physiological circumstances, helping the host to combat infections as part of first line host immune defence mechanisms. However, when activated systemically, particularly C5a is heavily involved in the pathological processes causing the clinical syndrome sepsis (42,49).

1.4 Toll-like receptors

The TLR family is constituted by a complex group of PRRs which recognize a wide variety of evolutionary conserved molecular structures (50). The main function of the TLRs is signalling to the cells as microbial PAMPs are recognized as exogenous danger (10). Over the last years it has however become clear that TLRs in addition sense endogenous ligands, which are cellular components of self-origin such as proteins and peptides, polysaccharides and proteoglycans, nucleic acids and phospholipids. Accumulating evidence demonstrates that endogenous ligand-mediated TLR signalling plays a role in several disease processes including tissue injury, repair and regeneration, autoimmune diseases and tumorigenesis – all conditions involving sterile inflammation (51).

To date, ten different TLRs have been identified in human (52). Concerning the exogenous ligands, each TLR has specificity for distinct PAMPs from bacteria, mycobacteria, fungi, parasites and viruses: Lipoproteins are recognized by TLR1, 2 and 9; double-stranded RNA by TLR3; LPS by TLR4, flagellin by TLR 5, single-stranded RNA by TLR7 and 8; and DNA

by TLR9 (52). Upon recognition of these PAMPs, TLRs recruit a specific set of adaptor molecules, such as MyD88 and TRIF, to induce downstream signalling (53).

In Gram-negative inflammation, LPS is the main inflammation inducing molecule, and TLR4 is considered to be the only LPS-receptor (54). TLR4 has therefore been regarded a possible interventional target for modulating the inflammatory response in Gram-negative induced inflammation. In order to induce downstream intracellular signalling by recognition of LPS, TLR4 is however dependent on cofactors. One of the cofactors, myeloid differentiation factor 2 (MD-2), is a soluble protein present on the extracellular domain of TLR4 and confers responsiveness to LPS (55). CD14, another TLR4 cofactor, was actually discovered before TLR4 itself; it was found to bind the circulating LPS in complex with LPS-binding protein (56,57). Upon binding with LPS, the CD14/LPS complex binds to the MD2/TLR4-complex and induces activation of intracellular signalling via the adaptor molecules MyD88 and TRIF to finally activate the nuclear transcription factor, which is responsible for transcription of a wide variety of inflammation associated genes and subsequent synthesis and release of several pro-inflammatory cytokines (53,58,59). Some Gram-negative bacteria such as *N. meningitides* induce signalling via both adaptor molecules, whereas *E. coli* predominantly induce signalling via MyD88 and *Salmonella* predominantly via TRIF (60).

As mentioned, TLR2 is the Gram-positive PRR analogue to TLR4 for Gram-negative bacteria (61,62). However, debate is still ongoing about the Gram-positive TLR2 ligand. Lipoteichoic acid (LTA) has been claimed to be the Gram-positive LPS analogue (59,60), but later studies have shown that especially LTA of *S. aureus* and *S. pneumoniae* are not ligands for TLR2, but in fact for the lectin pathway (63). On the other hand, the staphylococcal lipoproteins, Pantone-Valentine toxin and Phenol Soluble Modulins have been identified as potent TLR2 ligands, and little controversy exists over the statement that also TLR2 mediates Gram-positive inflammation (64). The TLR2 signalling is partly analogous to TLR4 signalling as TLR2 also uses a MyD88-dependent signalling pathway to translocate NF- κ B into the nucleus with subsequent activation of the expression of pro-inflammatory cytokine genes (64). Even more interestingly, it has been shown that TLR2 signalling by these staphylococcal ligands is CD14 dependent (65). The true promiscuous role of CD14 as co-receptor for the majority of

the TLRs, makes it an important molecule in the TRL family and an utterly interesting target molecule for anti-inflammatory purposes (50).

TLR2 and TLR4 are transmembrane lipoproteins located on the extracellular surface of the immune cells, but several other TLRs, including TLR3, 7, 8 and 9, are located on the inside of the cell in order to detect PAMPs and DAMPs intracellularly. In the setting of an infection, they are designed to and evoke an inflammatory response upon recognition of intracellular microorganisms including viruses and certain bacteria and parasites (17,66,67).

TLR stimulation is finally responsible for the production of a wide variety of pro-inflammatory cytokines, including IL-6 and TNF, which drive the inflammatory response (66,68).

1.5 Cross-talk: Complement, TLRs and coagulation

There is increasing evidence for a close and widespread cross-talk between complement and TLRs, but also between innate immunity and coagulation (69-71). Phagocytosis of mycobacteria and *Borrelia burgdorferi* has been shown to be enhanced by cross-talk between complement receptor 3 (CR3) and CD14, and killing of *Salmonella* has been documented to be dependent on TLR4 and CR3 (72-74). Several PAMPs, including the TLR4-ligand LPS and TLR2/6-ligand zymosan have been demonstrated to markedly increase the level of the pro-inflammatory cytokines IL-6, TNF and IL-1 β in a complement dependent manner (75). In the setting of septic inflammation, especially the proposed ability of C5a and TLRs to reciprocally promote pro-inflammatory cytokine production is of great relevance (75,76).

Innate immunity and coagulation are intimately connected and may be viewed as partners in the inflammatory response with the main goal of stabilizing an organism which has encountered a threat to its homeostasis (71). In settings where the complement system is activated, such as during an infection, the coagulation system is also tilted towards a pro-coagulant state (77). Similarly, whenever the coagulation system is triggered, for example

during trauma, an associated activation of inflammation will occur as the host is at risk of infection (78). This simultaneous activation of haemostasis and inflammation is in many clinical circumstances beneficial. For example, in the instance of an infection, the local formation of thrombi in the microvasculature draining the site of the infection supplies an important barrier which prevents the bacteria from entering the circulation.

In fact, not only are these systems concurrently activated. At various levels of inflammation and haemostasis, members of both systems interact directly or via secondary mediators. There are several examples of such interactions: C5a may directly trigger tissue factor (TF), the spark plug of the coagulation cascade; anticoagulation mechanisms are inhibited by binding of C4b-binding protein to Protein S, an important co-factor for degradation of coagulation factors Va and VIIIa via activated protein C (79); C3a and C5a promote the production of TNF and IL-6, which in turn enhance TF expression on monocytes and increase platelet thrombogenicity; lastly, observations *in vitro* also suggest that complement components may be cleaved directly by thrombin, plasmin and factor XIIa (71). Therefore, when studying inflammation, concurrently recording markers of haemostasis, systemically as well as in organs, provide important additional information.

1.6 Sepsis: Complement and TLRs

Under physiological conditions complement and TLRs act partly systemically by signalling danger, but predominantly promote local effects and as part of host defence mechanisms work to stop microorganisms from entering the circulation and strive after homeostasis. However, under pathological conditions, such as in sepsis, the tight regulation of these systems fail and systemic activation occurs promoting SIRS throughout the body, also in organs far from the site of danger (80-83). The favourable and essential local actions of innate immunity become the main threat to the host. For example, increased vascular permeability, capillary leakage and vasodilation are advantageous innate immunity effects in order to allow sequestration of neutrophils to the site of infection, but when these effects are promoted systemically, the net result is loss of intravascular volume, hypotension and reduced blood flow to vital organs in addition to organ inflammation upon sequestration of neutrophils to organs far from the site of danger (84,85).

The mentioned advantageous interplay between coagulation and innate immunity also becomes harmful in systemic infection-induced inflammation as haemostasis is tilted toward a more procoagulant state, promoting disseminated intravascular coagulation (DIC) (86). Hallmarks of DIC are the microvascular depositions of fibrin, platelet consumption and formation of microthrombi, also far from the site of infection, contributing to organ dysfunction and failure (86,87). The onset of DIC in sepsis has the clinically devastating importance of increasing mortality two-fold (88).

The overall myriad of inflammatory responses in sepsis is impossible to fully account for. In addition to complement, TLRs and coagulation, also the fibrinolytic system and the kallikrein/kinin system are activated (89,90). The endothelial cells are also important players in promoting dysfunctional microcirculation as adhesion molecules are upregulated and platelets and leukocytes adhere to the microvascular lining (91,92).

1.7 Sepsis treatment

To date treatment of severe sepsis and septic shock basically still relies on three arms: Initial resuscitation, anti-microbial therapy and supportive therapy.

The main goal of the first arm, initial resuscitation, is to re-establish adequate perfusion to all vital organs. Briefly summarised, haemodynamic support according to Guidelines from 2012 includes (2): initial fluid resuscitation with crystalloids and eventually albumin if needed; fluid challenge to hypovolemic patients; vasopressor support with noradrenalin and eventually adrenalin to maintain blood pressure above 65 mmHg.

The main goal of the second arm of treatment, anti-microbial therapy, is obvious and according to the above mentioned Guidelines from 2012 includes: taking blood cultures or cultures from the relevant site of infection before administering antibiotics in order to obtain a specific microbial diagnosis; broad-spectrum antibiotics until the microbe is known; extensive

investigations including imaging studies to obtain a certain source of infection; and lastly reassessment of microbial therapy daily and de-escalation of the microbial therapy according to microbiological results. Gaining control over the site of infection by surgically revising infected wounds, abscesses and so on is also of great importance.

The third arm, supportive therapy, is primarily aimed at helping the organs to maintain normal function and homeostasis, or secondarily replace malfunctioning organs. This includes mechanic ventilation, dialysis, sedation and administration of blood products and glucose. Use of corticoid treatment and prophylaxis against deep vein thrombosis and gastric ulcers are also recommended to specific patients groups when appropriate (2).

Given the elaboration above on innate immunity, inflammation and inflammatory mediators, a fourth arm of treatment in sepsis is missing and has long been sought: Mediator directed therapy. Several clinical trials blocking a range of mediators in sepsis including bradykinin, prostaglandins and platelet activating factor, and furthermore anti-thrombotic treatment using tissue factor pathway inhibitor and anti-thrombin have all failed to show beneficial effects (93-97).

IL-1 β and TNF are clearly linked to septic inflammation and both have been considered possible therapeutic targets in patients with severe sepsis (98-100). As the level of IL-6 in septic patients correlates with severity and mortality, IL-6 was also thought to be a possible therapeutic target (101,102). However, several large clinical trials show that increased survival has not been achieved by targeting these downstream cytokines selectively (103-106). There were also considerable expectations connected to treatment with recombinant human activated protein C (rhAPC), but promising results were not reproducible, and the ability to increase survival in a final multicentre study failed (107). Lastly, also the specific TLR4 inhibitor eritoran failed in clinical trials (108). In common, all these trials have tried to combat the inappropriate inflammation in sepsis by singling out one key downstream mediator. Given the inexplicably large number of downstream mediators flowing over as part of the inflammatory response, not to mention the cross-talk and redundancy of innate immunity, it is not surprising that these trials have failed.

Accordingly, the possibility of dampening the overall inflammatory load in sepsis seems more likely when targeting upstream. In this context targeting upstream means blocking down or at least slowing down the cascade mechanisms as close to the interaction between the PRRs and the PAMPs as possible. However, targeting each specific PAMP or its receptor would demand specific knowledge about the infecting microbe at the commencement of treatment, which in practice is impossible. Therefore, targeting unspecific pattern recognition receptors of several PAMPs may prove to be a possible way forward in the search for mediator directed therapy.

CD14 is one such molecule of the TLR family important for unspecific pattern recognition of several PAMPs, and the complement factor C3 and to a large degree also C5 another set of such upstream molecules acting as bottlenecks for downstream effects of the complement system. Indeed, inhibiting CD14 has been shown to have vast anti-inflammatory effects in septic pigs, and inhibition of complement factor C3 has also shown promising anti-inflammatory effects in septic baboons (109,110).

In 2008, following observations of improved anti-inflammatory effects when combining inhibition of TLRs and a key complement molecule, professor Mollnes and colleagues put forward the hypothesis that this combined inhibition may be a future strategy to attenuate the inappropriate inflammatory response seen in sepsis as well as in conditions with sterile induced inflammation (47,81). Several studies of the effect of the combined inhibition on sterile and non-sterile induced inflammation have been conducted since (111-115).

THE CONCEPT OF DUAL INHIBITION OF COMPLEMENT AND CD14

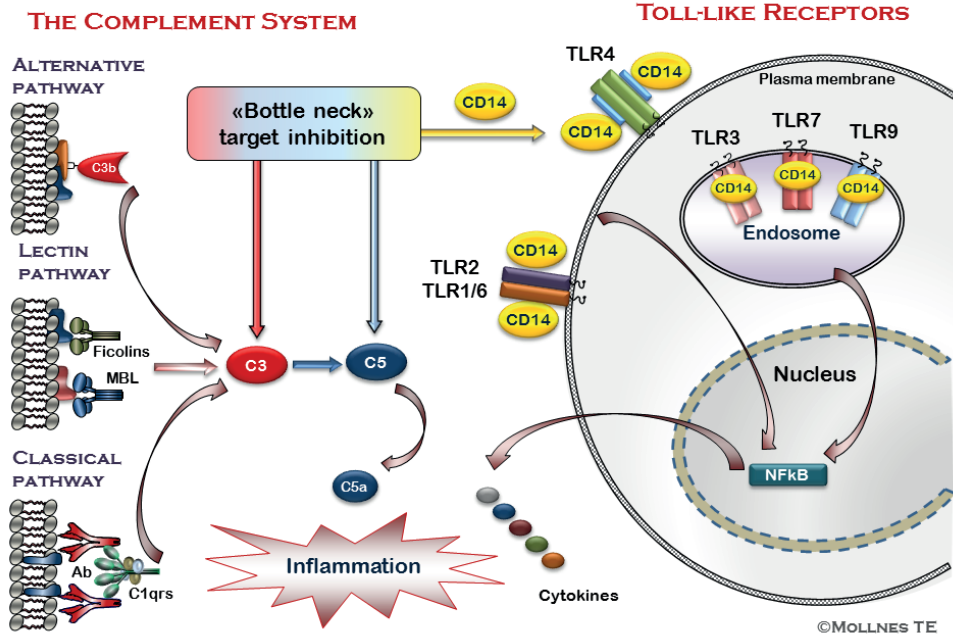


Figure 2. The concept of combined inhibition of complement and CD14 (with permission from the copyright holder T.E. Mollnes).

2. AIM

In this thesis, dissecting the effects of combined inhibition of a key complement molecule and CD14 under changing conditions of bacterial induced inflammation and sepsis has been the main task.

- A possible preservation of the anti-inflammatory effects of the combined inhibition of complement and CD14 was investigated under increasing concentrations of Gram-negative and Gram-positive bacteria *ex vivo* (Study **I**).
- The anti-inflammatory effects of the combined inhibition of C3 and CD14 when introducing a time delay between the onset of Gram-negative induced inflammation and the addition of the inhibitors were examined in the same *ex vivo* model (Study **II**).
- The effects on inflammation, haemostasis and haemodynamics of combined inhibition of C5 and CD14 were examined in a porcine *in vivo* model of *E. coli*-induced sepsis (Study **III**).
- Lastly, the efficacy of the combined inhibition of C5 and CD14 on local organ responses to septic inflammation was studied in the same *in vivo* model (Study **IV**).

3. MATERIALS AND METHODS

3.1 Bacteria

E. coli strain LE392 (ATCC 33572) and *S. aureus* Cowan Strain 1 (ATCC 12598) were obtained from American Type Culture Collection (Manassas, VA). Heat-inactivated *E. coli* was used in paper I and II, whereas live *E. coli* were used in paper III and IV. *S. aureus* (heat-inactivated) was used only in paper I.

3.2 Inhibitors

In paper I, azide-free mouse anti-human CD14 (clone 18D11; F(ab')₂ 3118, lot1383) was used in the *E. coli* experiments. The recombinant anti-human CD14 IgG2/4 Ab (r18D11) was used in the *S. aureus* experiments (116). For complement inhibition, either the complement C5 inhibitor, eculizumab (Soliris®) or the compstatin analogue Cp40, a complement C3 inhibitor, were used (117).

In paper II, azide-free mouse anti-human CD14 (clone 18D11; F(ab')₂ 3118, lot1383) and the Compstatin analogue AMY-90, a complement C3 inhibitor, were used.

In paper III and IV, recombinant bacterial *Ornithodoros moubata* Complement Inhibitor (OmCI), a 16.8 kDa protein also known as coversin (118), and mouse anti-porcine CD14 mAb (clone MIL-2, isotype IgG2b) were used.

3.3 Analyses

ELISA

In paper I and II, the terminal complement complex (TCC) was measured using a sandwich ELISA (119,120). Briefly, the monoclonal antibody (mAb) aE11 recognizes a neoepitope exposed in C9 after incorporation in the C5b-9 complex. aE11 was used as capture antibody binding to the soluble TCC. A biotinylated anti-C6 mAb (clone 9C4) was used as detection antibody. In paper I, the standard was normal human serum activated with zymosan and defined to contain 1000 arbitrary units (AU)/mL, whereas in paper II the standard was International Complement Standard #2 (ICS#2) (121), which is a standardized version of

zymosan activated normal human serum. Zymosan activated human serum was used as a positive control.

The granulocyte activation markers myeloperoxidase (MPO) and lactoferrin (LF) were quantified using commercially available kits from Hycult Biotech in paper **I**, whereas in paper **II** only MPO was measured.

In paper **III** Leukotriene B4 (LTB4) from serum was measured using a competitive enzyme immunoassay from R&D Systems. Citrate plasma was used for measuring thrombin-antithrombin (TAT) in an immunoassay kit from Dade Behring (122). Plasminogen activator inhibitor-1 (PAI-1) was measured in citrate plasma by a porcine PAI-1 kit from Molecular Innovations. The cytokines TNF, IL-1 β , IL-6 and IL-8 were analyzed by Quantikine Porcine Immunoassay kits from R&D Systems, whereas IL-10 was analyzed by a porcine kit from BioSource Invitrogen. A Complement System Screen (Wielslab®) from Eurodiagnostica AB was used to test functional activity of the classical, lectin and alternative complement pathways.

In paper **IV** IL-1 β was measured using the same kit as in paper **III**.

Multiplex analysis

In paper **I** and **II**, cytokines TNF, IL-1 β , IL-6, IL-8, MIP-1 α and MIP-1 β were measured using a multiplex cytokine assay from Bio-Rad Laboratories. In paper **III** and **IV**, TCC was measured using multiplex xMAP technology (123), and in paper **IV** the same technology was also used for measuring IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1). In short, antibodies for the biomarkers were coupled to carboxylated nonmagnetic beads using a Bio-Plex amine coupling kit from Bio-Rad. The nonmagnetic beads with the coupled antibodies were then incubated with organ homogenates or plasma samples and subsequently incubated with a secondary antibody followed by streptavidin-R-PE from Qiagen. Measurement and data analysis of all multiplex analyses were performed using Bio-Plex 100 System and Bio-Plex Manager software version 6.1.

Flow cytometry

Flow cytometry was used for measuring CD11b on monocytes and granulocytes in paper **I**. The cells were stained with anti-CD11b PE and anti-CD14 FITC. Flow cytometry was also used in paper **III**; the porcine ortholog to human CD11b, wCD11R3, was measured only on neutrophils *in vivo*, the cells stained with mouse anti-porcine wCD11R3 FITC. In the same study, TF on granulocytes was measured in separate *ex vivo* experiments, the cells stained with sheep anti-human TF and donkey anti-sheep IgG-FITC conjugate. In study **III**, haematological parameters including leukocyte differential count was measured on a routine hospital haematological instrument, which in principle is a flow cytometer. This instrument discriminated neutrophils in porcine blood, but monocytes and lymphocytes were not discriminated.

Histopathological evaluation

In paper **III**, two micrometre thick sections from formalin-fixed lung- and liver specimens were deparaffinised and stained by haematoxylin/eosin (H&E) and saffron. The lung biopsies were evaluated by morphometry in order to estimate the volume density of interstitial and alveolar inflammatory changes. In the liver biopsies granulocyte infiltration and oedema in the portal tracts were evaluated.

Quantitative real-time PCR analyses

In paper **IV**, homogenised tissue samples from harvested organs were analysed for gene regulation of C5a Receptor (CD88), TF, PAI-1 and IP-10 using TaqMan qPCR-primers.

3.4 Models

Ex vivo whole blood model

In study **I** and **II** a well-established whole blood model was used. The model was developed in order to maintain all functional effector systems open and able to interact, but without the blood clotting. The specific thrombin inhibitor lepirudin (Refludan®) leaves all functional effector systems but thrombin open to interact, and no interference with complement activation has been discovered (124). Although not equivalent to studying living organisms undergoing massive inflammation, the whole blood model allows studies of all inflammatory mediators and cross-talk between these systems.

Whole blood was drawn from healthy volunteering donors, directly anti-coagulated with lepirudin and further immediately processed. In paper **I**, all blood was preincubated for five

minutes with inhibitors or PBS before receiving increasing concentrations of either *E. coli* or *S. aureus* and then incubated for one hour (for measuring granulocyte activation markers) and four hours (for measuring cytokines and TCC) at 37 °C. For measuring CD11b, the blood was incubated for 15 minutes before the cells were fixed using formaldehyde and stained as described above. In paper **II** the inhibitors and PBS were added 5 minutes before or 5, 15 or 30 minutes after adding *E. coli*. The blood was incubated for one and two hours. The experiments were stopped using EDTA, and plasma was stored at -70 °C until analysed.

In vivo model

The *E. coli*-induced sepsis model which was used in study **III** and **IV** is previously well described with rapid increase of inflammatory and haemostatic biomarkers (125). The intravenous infusion of *E. coli* was conducted with a stepwise increase in bacterial concentration over four hours, whereby infusing a total of 1.075×10^8 *E. coli*/kg, which corresponds to 1.1×10^6 bacteria/mL blood. The pigs were extensively monitored using continuous ECG recording, artery line, central venous catheter and pulmonary artery catheter. The monitoring included recording of HR, MAP, MPAP, CVP, SaO₂ and urinary output. The insertion of a PiCCO-catheter was implemented in all experiments and allowed for measurement of stroke volume variation (SVV). Blood gases were measured regularly, and a pH of 7.40 was used as guide to adjust the respirator settings. Blood was also drawn regularly; at the beginning of the experiments, at the time the bacterial infusions were commenced and every hour thereafter. The blood drawn was used for analyses as mentioned above. At the end of the experiments, the animals were euthanized and organ samples were harvested immediately and transported on dry ice to be stored at -70 °C before further processing and analyses.

3.5 Statistical considerations

Parametric statistics were used in all studies. Whenever two groups were compared a *t*-test was applied. In study **II** parallel data were obtained from the same individuals and a paired *t*-test was used to compare each intervention group with the positive control. In study **IV**, the collected data were not paired; an independent sample *t*-test was used to compare the combined inhibition group with the positive control group.

Whenever multiple groups were compared, ANOVA was used and post-test analyses were applied. In study **I** one-way ANOVA followed by Dunnet's post-test was chosen to compare

single and combined inhibition with the positive control group. In study **II**, the same statistical analysis was used for comparison of single inhibition of complement and CD14 with combined inhibition. The positive control group was omitted from this testing as the intention of the analysis was to test whether the combined inhibition gave a significantly different result from the single inhibitors. In study **III** a two-way ANOVA followed by Bonferroni's correction for multiple comparisons was performed for analysing both intervention groups.

In study **II**, one might argue that it would be appropriate to correct for multiple comparison, as the combined inhibition was added and tested at multiple times after adding the bacteria. However, correcting for multiple comparisons under such circumstances would imply decreased power of the analyses for each time-point included in the analysis. Using correction for multiple comparison would therefore increase the likelihood of making type II errors and falsely maintain our null-hypothesis simply because too many time points of adding the combined inhibition were included in the analysis. Before doing any statistical testing, simple knowledge of the biological cascade systems under investigation tells us that the effect would be lost sooner or later. It was therefore considered appropriate to run *t*-tests to investigate at what time point the anti-inflammatory effect was lost.

A *P*-value < 0.05 was considered significant in all analyses.

4. SUMMARY OF THE MAIN RESULTS.

Study I

Heat-inactivated *E. coli* and *S. aureus* were added in increasing concentrations. In the *E. coli* experiments with combined complement and CD14 inhibition, IL-6 was significantly reduced at all bacterial concentrations, whereas the significant effect on IL-1 β , IL-8 and MIP-1 α was lost only at the highest bacterial concentration. For TNF and MIP-1 β , significant reductions were observed at the lowest bacterial concentration. Monocyte and granulocyte CD11b expression were significantly reduced at all bacterial concentrations. Lactoferrin was significantly reduced at the lowest bacterial concentration.

In the *S. aureus* experiments with combined complement and CD14 inhibition, IL-6 and TNF were significantly reduced at the lowest bacterial concentration and IL-8 was significantly reduced even with twice the bacterial load. Monocyte and granulocyte CD11b expression was almost completely abolished at all but the highest concentration of *S. aureus*; at the highest concentration it was still significantly reduced.

In conclusion, study I demonstrates well preserved anti-inflammatory effects of the combined inhibition, although with somewhat lesser effects on Gram-positive inflammation.

Study II

Anti-CD14, compstatin (C3 inhibitor) and the combination thereof were added 5 minutes prior to, or 5, 15 or 30 minutes after adding *E. coli*. Combined inhibition significantly attenuated IL-1 β , IL-8 and TNF when adding the inhibitors up to 30 minutes delayed. IL-6 was significantly attenuated when adding the inhibitors up to 15 minutes delayed, whereas MIP-1 α and MPO were significantly attenuated when adding the inhibitors 5 minutes delayed. Finally, as compared to selective inhibition of C3 or CD14, the combined inhibition significantly more effectively attenuated IL-1 β , TNF, IL-8 and MPO when preincubating the whole blood with the inhibitors. As compared to selective inhibition of C3, but not CD14, the combined inhibition significantly more effectively attenuated IL-6, MIP-1 α and MIP-1 β .

In conclusion, study II shows that the anti-inflammatory effect is gradually lessened and eventually lost as the time span between adding the bacteria and applying the inhibitors increase.

Study III

Thirty pigs were randomly allocated to a negative control group, a positive control group, or one of two intervention groups receiving either OmCI or OmCI and anti-CD14. In septic pigs, C5-inhibition completely abrogated complement activation and significantly attenuated the level of LTB₄. C5-inhibition alone significantly attenuated expression of TF on granulocytes, formation of thrombin-antithrombin complexes ($P<0.001$), and formation of TNF and IL-6 ($P<0.05$); the combined inhibition of C5 and CD14 completely abrogated or strongly inhibited the same biomarkers ($P<0.001$ for all). The combined inhibition also attenuated the formation of PAI-1 ($P<0.05$), IL-1 β and IL-8, increased the formation of IL-10, and completely abrogated the expression of wCD11R3 and the fall in neutrophil cell count ($P<0.001$ for all). With regards to haemodynamics, combined inhibition of C5 and anti-CD14 delayed the increase in heart rate by 60 min ($P<0.05$) and mean pulmonary artery pressure by 30 min ($P<0.01$).

In conclusion, study III clearly demonstrates that the combined inhibition exerts impressive and broad systemic anti-inflammatory effects in Gram-negative induced sepsis.

Study IV

Tissues from the same pigs used in study III were examined from the combined treatment group, the positive control group and the negative control group after four hours of increasing bacterial infusions. IL-1 β and IL-6 were significantly attenuated in liver, kidney, lung and spleen. IL-8 was significantly attenuated in kidney, lung, spleen and heart, whereas MCP-1 was significantly attenuated in liver, kidney, spleen and heart. The combined inhibition significantly attenuated tissue factor mRNA upregulation in spleen and IP-10 mRNA upregulation in heart, liver, spleen and kidney. Finally, C5aR mRNA downregulation was prevented in heart and kidney. The anti-inflammatory effects in lung and heart correlated with the delay in hemodynamic changes observed in study III.

In conclusion, study IV adds important information about the anti-inflammatory effects on organ-level, supporting the findings from study III, and indicating preserved organ function by the combined inhibition.

5. DISCUSSION

The four studies constituting this thesis explore and elucidate the efficacy of the combined inhibition of a key complement molecule and CD14 on bacterial induced inflammation and sepsis. The different papers demonstrate the efficacy from several perspectives. In paper **I**, the preservation of the anti-inflammatory effects is demonstrated over a broad range of Gram-negative and Gram-positive bacterial concentrations. In paper **II**, the effect when adding inhibitors delayed is demonstrated, in addition to statistically proving the superior anti-inflammatory effect of the combined treatment as compared with single inhibition of either complement or CD14. In paper **III**, the *systemic* effects of the combined inhibition *in vivo* are demonstrated exploring haemodynamics, inflammatory biomarkers and haemostasis in porcine sepsis. In paper **IV**, the *local* effects of the combined inhibition are demonstrated exploring inflammatory and haemostatic biomarkers in septic porcine organs. These findings are important contributions to “*proof of concept*” of such combined inhibition.

5.1 Upstream versus downstream inhibition

As mentioned in the introduction, severe sepsis is a state of whole-body inflammation triggered by an infection, a condition with high morbidity and mortality (103). The initial triggering of the inflammatory state involves numerous biological systems (80,89,90,126). Previously, sepsis related research has tried to single out one of several downstream inflammatory mediators appearing early in the reaction in order to dampen the inflammation. This has mainly been done on the basis that mediators such as TNF, IL-1 β , IL-6 and IL-8 are correlated with morbidity and mortality in sepsis (101,127-130). Despite promising experimental and preclinical results, increased survival has proven extremely difficult, not to say impossible to achieve on this basis (104-106). The inability of this approach to cause an overall reduction of inflammatory mediators has later been confirmed (131,132).

As downstream inhibition of single inflammatory mediators failed, targeting upstream bottlenecks of these biological systems in order to block several downstream inflammatory mediators and their secondary effects emerged as a possibility. The principle of this approach is to inhibit upstream as close to the interaction between PRRs and their ligands as possible. Increasing research material over the last decade has proven the principle of this approach, in particular when targeting complement or CD14, with net attenuation of several downstream

inflammatory mediators, such as TNF, IL-6, IL-8 and IL-1 β (109,110,114,133,134), but also with *in vivo* studies suggesting increased survival (135,136). The concept of upstream inhibition of inflammation is therefore well founded and has superior anti-inflammatory potential as compared with downstream inhibition.

The other side of the coin is however that such upstream inhibition of innate immunity may have adverse effects. One of the functions of the complement system is opsonisation for phagocytosis and clearance of bacteria (25,38). However, the problem with impaired opsonisation is solved by inhibiting downstream of C3, as cleavage of this molecule is the key to this function. In the case of Neisserial infections, the complement system is also responsible for lysis of the bacteria (38). Therefore, complete inhibition must be done with great care in order to avoid increasing the risk for new infections, rather than decreasing the unfavourable hyperinflammatory state observed in sepsis.

Experience with complement inhibition already exists with PNH-patients, and the risk for Neisserial infections is indeed increased (137). This increase is however shown to be manageable with vaccinations and under certain circumstances prophylactic use of antibiotics. Similarly, case reports of C5 inhibition with concurrent use of antibiotics and vaccinations in patients with catastrophic anti-phospholipid syndrome (CAPS) have proven clinical benefit without adverse infections (138). Importantly, the PNH-patients receive complement inhibition over an extended period of time, whereas in the case of proposed sepsis treatment, the complement inhibition would be applied short term, either prophylactically to patients at risk for developing sepsis, or to patients with early signs and symptoms of sepsis (81). Both patient populations should under all circumstances be treated with antibiotics concomitantly.

5.2 Single versus combined inhibition – redundancy of host defence.

Several studies inhibiting either a key complement molecule or TLRs in sepsis prove to have anti-inflammatory effects, but often with some residue productions of the downstream inflammatory mediators, such as the cytokines mentioned above (109,115,133,134). Taking the numerous biological inflammatory systems activated in the course of sepsis, redundancy of host defence inflammatory mechanisms is a plausible explanation for this, and the using the potential to increase the anti-inflammatory effect by combining inhibition of two of the major inflammatory systems is one possible approach to overcome this problem, as set forward in the hypothesis proposed by professor Mollnes and colleagues (81).

In paper **I** and **II**, redundancy of host defence inflammatory mechanisms were demonstrated several times. MPO was the most obvious example, where single inhibition of complement or CD14 barely affected the level of MPO, whereas the combined inhibition had a striking effect, thereby demonstrating not only additive but also synergistic anti-inflammatory effects of the combined inhibition. Although not all readouts displayed such synergistic anti-inflammatory effects, all readouts in paper **II**, and most readouts in paper **I** and **III** point to an increased anti-inflammatory effect by the combined inhibition. Especially in paper **I**, the robustness of the combined inhibition was tested, and in comparison with selective inhibition of complement and CD14, the combined inhibition achieved a strikingly more sustainable anti-inflammatory effect. In paper **II**, the anti-inflammatory effect of combined inhibition was tested against single inhibition and also proven to be statistically more effective. These observations are in line with previous and later research, in Gram-negative, Gram-positive and polymicrobial induced inflammation (111,112,115,139,140).

In all papers but paper **I**, *E. coli* is the sole inflammation inducer, and the model is therefore relatively LPS-driven and CD14 dependent (56). In paper **I**, *S. aureus* is also used for inducing inflammation, and this model is relatively more complement driven (63,111). Although only hypothetically, one might assume that complement inhibition in *S. aureus* sepsis and CD14 inhibition in *E. coli* sepsis were sufficient to achieve proper anti-inflammatory effect. However, acquiring a microbiological diagnosis is always time demanding and quite often ends up being inconclusive. The combined use of complement and CD14 inhibition would therefore still be of relevance (6,7). In further support of the combined inhibition in sepsis the microbiological diagnosis is often polymicrobial and thus both complement and CD14 should be inhibited. This being said, all data in this thesis indicate a clear anti-inflammatory benefit from the combined inhibition as compared with single inhibition, regardless of the causative bacteria being Gram-positive or Gram-negative.

5.3 Ex vivo studies

In the *in vitro* part of this thesis, one of the goals was trying to find new approaches to test the combined inhibition of complement and CD14 in an *ex vivo* sepsis model, simulating aspects of the clinical setting. Without any argument, *ex vivo* sepsis studies cannot ever become actual sepsis studies, but rather studies of bacterial induced inflammation. Although the whole blood model allows for all the components of the blood but thrombin to interact, the disadvantage is

that all other cell systems of the organism, including the sepsis relevant endothelium of the vascular system, are not part-takers in the induction of inflammation in this model. In addition, the blood is continuously exposed to air and plastic from the surface of the tubes, the latter being known to activate the complement system (112).

Nevertheless, paper **I** and **II** were attempts to mimic some of the features of live sepsis and application of possible treatment regimen. In paper **I**, the gradually increased bacterial load was meant to resemble the different stages of sepsis, and previous research, especially in meningococcal disease, show a clear correlation of bacterial load in the patients and the disease severity (141). From a clinical point of view, although being far from the bedside, we therefore found it interesting to test to what extent the effect of the inhibitors would be preserved. The obvious con is of course that the efficacy was tested whilst under otherwise non-clinical circumstances, the inhibitors were for instance added prior to the bacteria.

In the clinical setting, early onset treatment is of vital importance. To draw a comparison between antimicrobial treatment and mediator directed anti-inflammatory therapy, the sooner one may start treatment, the better and faster the response to the treatment. The likelihood of commencing antimicrobial treatment and anti-inflammatory treatment before the onset of the infection is small. Most of the research on complement and CD14 inhibition in sepsis has been conducted without delay between the onset of inflammation and the adding of complement- or CD14-inhibitors. What are really being tested under such circumstances are “*proof of concept*” and the efficacy of the inhibitors when used prophylactically. The main point of paper **II** was to test the ability of the combined inhibition to attenuate inflammation, also when adding the inhibitors after triggering the inflammatory mechanisms. The results which show that the effect was gradually lost with increasingly delayed addition of inhibitors, were not unexpected and underscore that in a possible future mediator directed therapy, aiming for “the sooner the better” would still be the only suitable approach.

5.4 *In vivo* studies and the pig as model animal

In vivo animal models are substitutes for real patients; however such models approximate live sepsis as closely as possible in preclinical research. Mice have been, and still are, the most used model animal for studying sepsis, and mice as model animal for sepsis research are still of great relevance. However, mice are not men and there are obvious disadvantages with the use of mice in sepsis research (142). In most aspects pigs are relatively more suitable animals

to study human sepsis as they have the advantage of displaying closer physiological, anatomical, genomic and immunologic resemblance to human (143,144). For these and practical reasons, the pig was chosen as the animal model in our studies. The pigs used in our experiments were only a few weeks of age and the size of the pigs was approximately 15 kg. It is therefore probably more correct to draw lines between this model and paediatric sepsis as both size and immunologic maturity will influence the inflammatory response (145).

In a porcine sepsis model for human disease, awareness of pig peculiarities is also important; in particular the resident pulmonary intravascular macrophages are challenging (146). They resemble human liver Kupffer cells, engulf circulating microparticles including LPS of *E. coli* and constitutively express TLR4, which produce large amounts of proinflammatory cytokines such as IL-1 β upon stimulation with LPS (146,147). The immunoreactive features of the porcine lung may explain the extremely rapid increase in MPAP caused by the intravenous infusions of the bacteria, a problem not commonly encountered in septic human patients.

Investigating inflammatory mediators and the attenuation of such mediators by intervention is a common and relevant approach in sepsis research. One of the great advantages of *in vivo* studies is the ability to investigate relevant physiological parameters. Normalisation or at least improvement of cardiac and pulmonary function upon intervention in sepsis gives greater prospects of actual clinically significant effects as compared to net reduction of a single cytokine. In study **III**, the significant delay in haemodynamic changes is therefore of great interest. Furthermore, the link between reduced haemodynamic changes in study **III** and reduced pulmonary and cardiac inflammation observed in study **IV** strengthen the hypothesis that the combined inhibition may improve the function of vital organs in sepsis, whereby reducing the risk for multiple organ failure. As the number of failing organs is correlated to mortality in sepsis, it is of course tempting to speculate that the anti-inflammatory effect may also reduce mortality (6).

On the other hand, one might argue that the duration of the delay in haemodynamic changes in study **III** was not particularly prolonged. However, the intravenous *E. coli* sepsis model used in our study was extremely forceful, and the piglets received bacterial loads leaving them on the brink of haemodynamic collapse within a very short period of time. The above described problems with increased MPAP also forced us to use relatively large amounts of

norepinephrine, and one might speculate that the delay in haemodynamic changes would have been more prolonged were it not for the use of norepinephrine.

The model used in the study partly resembles the clinical picture of meningococcal disease with *N. meningitides*. However, there is no such thing as a standardized course of sepsis (148), and the vast majority of septic patients do not display such a rapid development of the disease (149). A less forceful model, such as the polymicrobial cecal ligation and puncture (CLP) model, would therefore likely be able to show a more prolonged delay in haemodynamic changes, and one might argue that a less forceful model would be preferable and maybe better suited also to possibly demonstrate increased survival (149). In support of this view, a mouse CLP study demonstrated increased survival by combined inhibition of CD14 and complement (139), and interestingly survival was also shown to be significantly increased as compared with the selective inhibition of complement and CD14.

5.5 The relevance of readouts

In sepsis, a “cytokine storm” is released as a response to the initial interaction between PAMPs and PRRs (150). All readouts in the papers were carefully selected with respect to their relevance in sepsis. TNF and IL-1 β are known to be part of the early inflammatory response, whereas several other cytokines such as IL-6 and IL-8 are induced slightly later (18,151). These cytokines are key inflammatory mediators and have been considered possible therapeutic targets for intervention in sepsis. They have been shown to increase with morbidity and mortality in sepsis, and a decrease has been shown to correlate with the resolution of sepsis (98,100,105). Therefore, the broad net reduction of these inflammatory biomarkers in all papers constituting this thesis is of great relevance trying to explore the anti-inflammatory potential of the combined inhibition.

The soluble terminal complement complex (TCC) is another biomarker examined in all papers. When using a complement inhibitor as part of the intervention, the relevance of detecting soluble TCC is obvious as a massive reduction of TCC confirms that the complement inhibitor works in the model. Secondly, and especially interesting in the *in vivo* sepsis model, TCC has been shown to correlate with mortality in sepsis, at least in meningococcal disease (152), and changes in TCC therefore may also convey possible clinical relevance. TCC has readily been detected *in vitro* using ELISA, so also in paper **I** and **II**, but in *porcine* plasma samples, we have previously been unable to detect TCC. The explanation

for this is probably that the antibody used for detecting TCC in porcine plasma is based on cross-reactivity, but the sensitivity for porcine TCC is only moderate as compared to human TCC (120). In study **III** and **IV** we were able to access newly developed multiplex methodology whereby increasing the sensitivity for porcine TCC (123). Detection of TCC in plasma and organs was therefore an encouraging achievement in itself. The increase of TCC in plasma and tissues from the positive control group confirms the view that complement activation contributed to the inflammatory response in the *in vivo* model. In addition we may speculate that the significantly reduced levels of TCC in plasma and tissues from the animals receiving OmCI convey a possible clinical relevance of complement inhibition.

The cross-talk between innate immunity and coagulation and fibrinolysis also makes relevant the examination of markers of coagulation and fibrinolysis (71,153,154). The convincing effect on TAT, PAI-1 and TF in study **III**, and TF and PAI-1 in spleen in study **IV** opens the possibility for reducing the risk for developing DIC by the combined inhibition; potentially of great significance given the clear relationship between development of DIC and mortality in sepsis (86-88).

5.6 Future perspectives

Paper **III** showed the effect of combined inhibition of complement and CD14 *in vivo*, and the effect was observed also in porcine organs in paper **IV**. These studies are preclinical “*proof of concept*” studies and importantly demonstrated that this kind of intervention is worth further studies. The prophylactic use of mediator directed inhibitors is so far not on the agenda in sepsis treatment. It may however be possible to find patients at risk for developing sepsis, and treat them prophylactically, but this remains a question for the future.

Paper **II** explored ways to simulate the clinically relevant delay between the initial triggering of the inflammation and the intervention with complement and CD14 inhibition. More importantly the study opened the possibility for a therapeutic window of time, and as such this study constitutes a step towards more clinically relevant testing of the concept. Conducting an *in vivo* study, much like study **III**, but with delayed administration of the combined inhibition would be a next step in the hunt for possible clinical application of such treatment.

Interestingly, a previous post-challenge study on the effect of an anti-C5a antibody in a mouse CLP-model demonstrated increased survival (155). Testing the concept of post-challenge combined inhibition of complement and CD14 in large animals using other sepsis models

such as the polymicrobial CLP-model, and also in models using other microbial agents such as *S. aureus*, would also be of great interest.

As mentioned in the introduction, DAMPs and PAMPs appear largely to depend on the same PRRs to evoke inflammatory responses (9). Translational value of research on pathogen-induced inflammation to sterile-induced inflammation may thus prove to be of significance. Research from our own group show that treatment with OmCI significantly reduced infarction size and improved cardiac contractility and function in a porcine model of cardiac ischemia-reperfusion injury (personal communication with Søren E. Pischke). This finding is supported by existing research on the role of complement in ischemia-reperfusion injury (156,157). Even more interestingly, also TLR4 and TLR2 are shown to be implicated in this sterile inflammatory process (158,159), and expectations are attached to the testing of combined complement and CD14 inhibition in a similar ischemia-reperfusion model. As ischemia-reperfusion injuries also occur in several other conditions such as cerebral infarction and transplantation, the possible clinical value of such research may prove to be substantial.

6. CONCLUSIONS

This thesis clearly demonstrates that combined inhibition of complement and CD14 effectively attenuates Gram-negative and Gram-positive bacterial induced inflammation, even when challenged with relatively high bacterial concentrations. Furthermore, the significance of delay between the initial triggering of inflammation and intervention is explored, and the results points to a possible therapeutic window of time. The anti-inflammatory effect of the combined inhibition of complement and CD14 on Gram-negative induced inflammation is also shown to be statistically superior to selective inhibition of complement as well as CD14. The anti-inflammatory effect of the combined treatment is demonstrated *in vivo* on a broad range of clinically relevant inflammatory, haemostatic and haemodynamic parameters. Lastly the anti-inflammatory effect of the combined inhibition is demonstrated also in organs. The correlation between the anti-inflammatory effects in organs and the delay in haemodynamic derangement observed, underscores possible future clinical relevance of the combined inhibition of complement and CD14.

It is difficult to draw hard conclusions upon relatively small amounts of data. However, altogether the four studies prove the anti-inflammatory *potential* of the combined inhibition of complement and CD14, in Gram-negative and Gram-positive induced inflammation. The results of the *in vivo* studies in particular point to a potentially clinically relevant application of the combined inhibition of complement and CD14, although it is known that a lack of correlation between animal studies and clinical trials is not uncommon.

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